Isolation of a factor from cotton leaf that recognizes *Beijerinckia*

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The selective retention of *Beijerinckia*, a nitrogen-fixing bacterium on cotton leaf surfaces was shown to be due to a binding factor. Gel diffusion and inhibition studies suggested that the factor from the leaf binds to lipopolysaccharide of the bacterium. SDS–polyacrylamide gel electrophoresis of the purified factor gave a single band corresponding to M_r ~19500. The organism showed agglutination activity with the factor when it reached stationary phase of growth. A positive correlation between encapsulation of the organism and agglutination activity was also observed.

Beijerinckia-*binding factor* *Cotton leaf* *Lectin* *Agglutination* *Lipopolysaccharide* *Capsule*

1. INTRODUCTION

The ability of plants to respond to the presence of pathogenic or symbiotic micro-organisms is an important aspect of their physiology. Several recent studies have indicated that the carbohydrate-binding plant proteins known as lectins might function in the recognition of microbial pathogens and symbionts by binding to characteristic carbohydrate receptors on the microbial cell surfaces [1–6].

Beijerinckia was found to be the lone nitrogen-fixing micro-organism occurring on the leaf surfaces of cotton plants. While there is a good deal of evidence to support lectin involvement in the legume–*Rhizobium* associations, no experimental evidence is so far available on 'loose' associations such as the phyllosphere. Preliminary observations that cotton leaf crude extracts strongly agglutinate *Beijerinckia* cells prompted me to suggest a possible involvement of a binding factor similar to lectins reported in the legume–*Rhizobium* interactions [7]. I report here the isolation of the leaf factor and its interaction with the bacterium.

Abbreviations: BBF, *Beijerinckia*-binding factor; EPS, exopolysaccharide; LPS, lipopolysaccharide; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate

2. MATERIALS AND METHODS

2.1. *Chemicals*

Chemicals required for the purification of the factor and the bacterial polysaccharides were purchased from Sigma Chemicals (St Louis MO). All other chemicals were of analytical grade.

2.2. *Organism and growth conditions*

The strain of *Beijerinckia* isolated from cotton (*Gossypium hirsutum* L. var. Varalaxmi) leaf surfaces was grown in nutrient broth [7] by incubating at 30°C on a rotary shaker (120 rev./min). Unless otherwise mentioned, cells from stationary phase of growth (4-day-old) were used throughout.

2.3. *Agglutination assays*

The bacteria were harvested, washed thrice with phosphate-buffered saline (PBS) (100 mM K_2HPO_4/KH_2PO_4, 150 mM NaCl, pH 7.2) and resuspended in the same medium. The agglutination reactions were performed [7] in agglutination plates with 2-fold dilutions of the factor in PBS,

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keeping bacterial concentration (~10^8 cells/ml) constant throughout. The incubations were carried out at room temperature (28°C). One unit was defined as the highest dilution of the factor at which agglutination is still visible.

2.4. *Isolation of cell surface polysaccharides*

Exopolysaccharide was precipitated from the culture supernatants by using cetyl pyridinium chloride and lipopolysaccharide was extracted from the bacterial cells with phenol–water extraction procedure as in [8].

2.5. *Gel diffusion*

The gels were prepared with agarose (0.7%) in 80 mM veronal buffer (pH 8.6). The plates after the additions were incubated at room temperature for 12–24 h and examined for precipitin lines.

2.6. *Transmission electron microscopy (TEM)*

TEM was carried out with whole mounts of *Beijerinckia* cells from different stages of growth after staining with ruthenium red as in [7].

2.7. *Purification of the binding factor*

Crude extracts were prepared with healthy leaves (300 g wet wt) from flowering stage plants in PBS containing 50 mM of sodium ascorbate as in [7]. The nucleoproteins from the concentrated crude extract were precipitated with 1 M $MnCl_2$. The supernatant was desalted by passing through a Sephadex G-25 column (1 × 25 cm). The supernatant from the previous step was loaded on a DEAE-cellulose column (1.4 × 20 cm) equilibrated with 100 mM phosphate buffer (pH 7.2). Elution of the adsorbed protein was carried out by a linear gradient of 0–1 M NaCl in the same buffer. Fractions showing agglutination activity were pooled, dialysed against PBS and concentrated. All the operations were carried out at room temperature. The protein content was determined by turbidimetric method [9] using 50% trichloroacetic acid.

2.8. *Gel electrophoresis*

Electrophoresis was performed with 6.5% acrylamide gels using 50 mM Tris–glycine buffer (pH 8.3) or 50 mM glycine–acetic acid buffer (pH 4.0) [10,11]. SDS–polyacrylamide gel electrophoresis was done as in [12].

3. RESULTS

3.1. *Interaction between* Beijerinckia *surface polysaccharides and cotton leaf factor*

A double diffusion test for cotton leaf crude extract against LPS and EPS of *Beijerinckia* is shown in fig.1. A precipitin line was observed between LPS and the leaf extract, while the EPS failed to react with the extract. When a non-homologous protein such as BSA (1 mg/ml PBS) was used in place of leaf extract, the former did not react either with LPS or EPS suggesting that the factor in the leaf might be specific to LPS of the organism. This observation was further confirmed when the leaf extract preincubated with LPS (5 mg/ml) for 1 h failed to agglutinate the bacterium. Similarly, addition of LPS to the preagglutinated cells resulted in the reversion of agglutination reaction. Furthermore, there was ~95 ± 1% (mean ± SD, 5 expt.) inhibition in the adsorption of ^{14}C-labelled *Beijerinckia* cells to the surface when the leaves were pretreated for 1 h with LPS (not shown). The precipitation of extract with LPS and the inhibition of agglutination and adsorption of ^{14}C-labelled *Beijerinckia* to leaf surface by LPS indicated that the externally added LPS might have acted competitively so that further binding of the organism to the factor was inhibited suggesting that the LPS of the organism had the receptors for

Fig.1. Ouchterlony double diffusion showing the interactions between cotton leaf extract and the cell wall polysaccharides of *Beijerinckia*: (centre) leaf crude extract (2 mg protein/ml); (1) PBS; (2) LPS (1 mg/ml); (3) EPS (5 mg/ml).

the binding factor. These inhibition studies with LPS also ruled out the possible artifacts due to biochemically nonspecific interactions.

3.2. *Purification of the binding factor*

The extract after precipitation of nucleoproteins with $MnCl_2$ showed a 5-fold increase in specific activity (agglutination activity) over the crude extract (table 1). (The ammonium sulfate precipitated leaf proteins, however, lost the agglutination activity. The addition of divalent metal ions to the precipitate did not help restore the activity.) The supernatant from the above step when

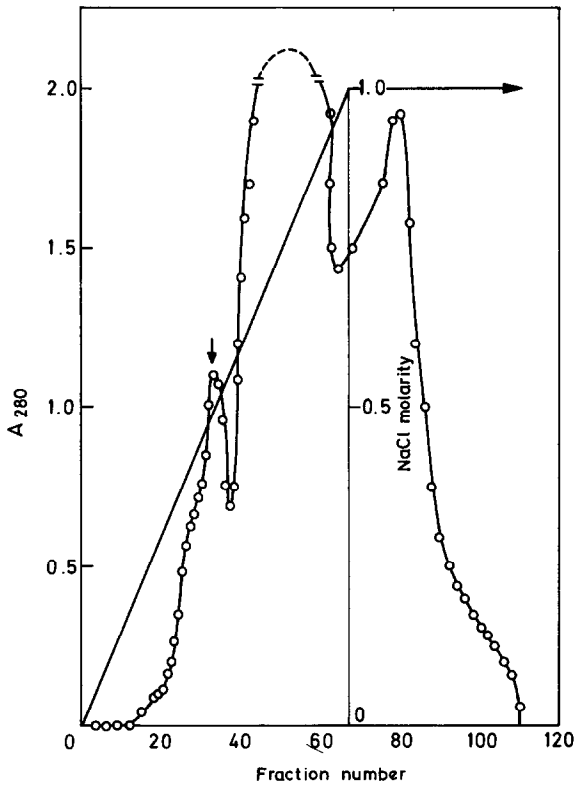

Fig.2. (a) DEAE-cellulose column (1.4 × 20 cm) chromatography of cotton leaf extract for the purification of BBF. The supernatant from $MnCl_2$ precipitation step was desalted on Sephadex G-25 column and loaded onto a DEAE-cellulose column. A linear gradient of 0–1 M NaCl in phosphate buffer was applied and 1.5 ml fractions were collected. The linear gradient was used up to 67 fractions and the remaining were collected with 1 M NaCl in the same buffer. Active fractions (vertical arrow) were pooled and dialysed against PBS.

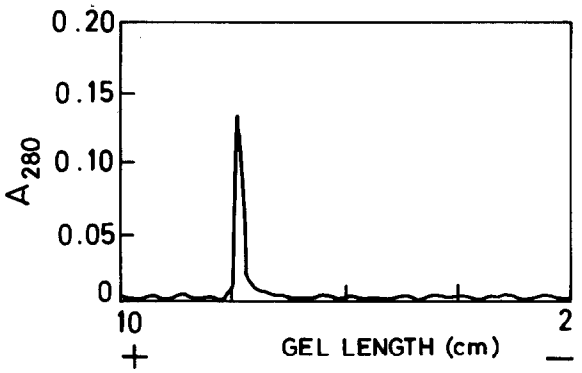

Fig.2. (b) SDS–polyacrylamide gel (6.5%) electrophoresis of BBF. Sample in 62.5 mM Tris–HCl buffer (pH 6.8) containing 2% SDS, 10% glycerol, 5% mercaptoethanol and 0.001% bromophenol blue was boiled for 3 min and the electrophoresis was carried out using 25 mM Tris–glycine buffer (pH 8.3) containing 0.1% SDS. The gel was fixed in 12.5% trichloroacetic acid for 2 h and scanned at 280 nm. (The standard markers were prepared in a similar way, but stained with Coomassie brilliant blue R.)

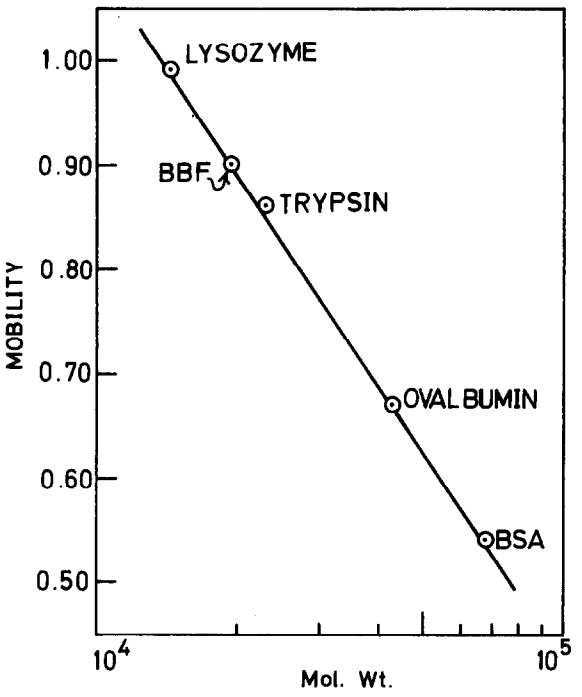

Fig.2. (c) Determination of BBF M_r by SDS–polyacrylamide gel electrophoresis. The standard markers used were (kDa): lysozyme (14.3); trypsin (23.3); ovalbumin (43); BSA (68). The markers' M_r-values were plotted against mobility on a semilog graph. The M_r of the factor was determined from the standard graph by calculating its mobility on the gel.

Table 1

Purification of *Beijerinckia*-binding factor from cotton leaf

Step no.	Fraction	Protein (mg/ml)	Agglutination (units)	Spec. act. (units/mg protein)	Purification (-fold)
I	Leaf crude extract	25.000	20	0.8	0
II	Supernatant after $MnCl_2$ precipitation and desalting on Sephadex G-25 column	5.160	20	3.9	5
III	DEAE-cellulose peak I protein (0–1 M NaCl linear gradient)	0.018	10	555.5	694

Yield = 220 μg

chromatographed on DEAE-cellulose column, gave 3 protein peaks on elution with NaCl (fig.2a). The agglutination activity was recovered in the first peak eluting out at 0.40–0.55 M NaCl. The purification procedure employed here resulted in preparation of ~694-fold purified factor over the crude and the yield was ~220 μg (table 1). The pooled active fractions were dialysed, concentrated and subjected to polyacrylamide gel electrophoresis. The factor failed to take up either Coomassie blue or basic fuschin stains and hence the gel was scanned at 280 nm. The factor did not move in the gel at alkaline pH. However, under acidic pH, the factor moved considerably (not shown). The factor on SDS–polyacrylamide gel showed a single, prominent peak at 280 nm (fig.2b) and the comparison of its mobility with the standard markers on the gel revealed an M_r ~19500 (fig.2c).

3.3. *Agglutinability of* Beijerinckia *with the factor as a function of growth phase*

Highest agglutination activity was observed around 80 h when the culture was in its stationary phase of growth and the capsule was prominent (fig.3). No agglutination could be observed when the cells were in exponential phase (up to around 40 h) or beyond 100 h in the stationary phase. Interestingly, no capsule was evident at these stages as revealed by TEM (fig.3).

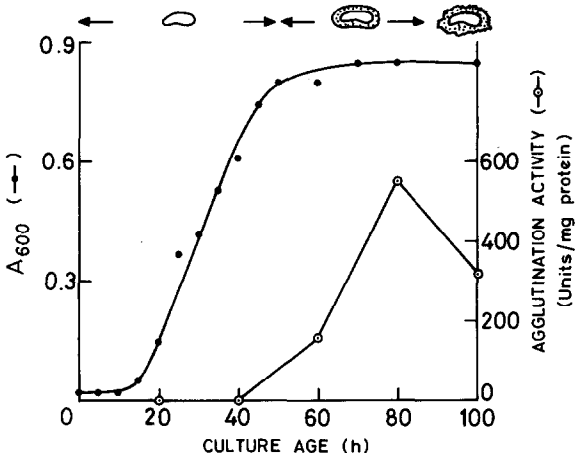

Fig.3. Agglutinability of *Beijerinckia* with the purified factor as a function of growth phase. The formation and disintegration of capsule with culture age as revealed by TEM has been diagrammatically represented at the top.

4. DISCUSSION

This study implicates a binding factor in the leaf–microorganism interactions. The factor in the leaf extract reacted with LPS of the organism in the double diffusion tests. Binding of LPS from various species of *Rhizobium* to lectins of their host legumes has been reported [8,13,14]. Agglutination activity of *Beijerinckia* with the factor was observed only when the cells reached sta-

tionary phase of growth. Highest percentages of soybean lectin-positive cells have been reported from the early and midlog phases of growth in *R. japonicum* [4]. However, it has also been shown that the appearance on the surface of *R. trifolii* of determinants to clover lectin binding starts only as cells left lag phase and as they entered stationary phase of growth [15]. The somatic O antigen of *Salmonella* LPS was reported to have a stronger expression in stationary phase than in exponential phase (see [15]).

In addition, a positive correlation was also observed between the encapsulation of *Beijerinckia* and the agglutination reaction with the factor. The factor, however, did not react with the capsule forming EPS of the organism in gel diffusion tests. A combination of the change in capsular composition and loss of encapsulation accounting for the loss of lectin binding capacity of *R. japonicum* strains has been indicated [16]. Dual binding sites for peanut lectin on the rhizobial cells have also been reported with EPS serving as the major lectin binding component and LPS probably offering the secondary site [17]. Whether such a phenomenon is true with *Beijerinckia* also is, however, not known. Gel diffusion and inhibition studies with ^{14}C-labelled *Beijerinckia* in the present investigation provide more support to indicate that LPS is important in the leaf–*Beijerinckia* interactions.

These observations suggest a possible molecular basis for the selective retention of *Beijerinckia* on cotton leaf surfaces. However, the present results raise a number of questions. How specific is the factor to *Beijerinckia*? My experience with *Azotobacter vinelandii* indicated that this organism could be weakly agglutinated by the leaf extracts though this bacterium does not occur on the leaf surface. What does the capsule do? Which sugar(s) in the LPS acts as the 'receptor' for the binding factor? Do such factors exist in other plant species? Answers to such questions should contribute toward the rational extension of symbiotic nitrogen fixers to non-leguminous crop plants of agricultural importance.

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